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13. ABSTRACT (Maximum 200 words)

The objectives included the development of new diagnostic procedures and vaccine strategies pertinent to selected phleboviruses. Using cDNA clones of particular phlebovirus genes, two eukaryotic expression systems were studied. Recombinant vaccinia viruses were made that expressed part of the Punta Toro (PT) M RNA, producing the PT glycoproteins G1 and G2. The expressed products were characterized. Baculovirus vectors were developed and employed for the expression of PT S RNA gene products (N and NS proteins). Baculoviruses were also used to express Hantaan S RNA (N protein) and the product tested a potential diagnostic reagent. A second objective involved the characterization of an attenuated, candidate vaccine derivative of Rift valley fever (RVF) phlebovirus. The complete sequence of the viral M RNA of the virus was obtained, the encoded glycoproteins expressed in baculovirus vectors and the products characterized. The sequence of the RVFV S RNA was obtained and compared with those of TOscana (TOS) phlebovirus, PT virus, Sicilian sandfly fever virus and Uukuniemi virus. The homologies of these sequences identified conserved and variable regions of the genes and their products. The S gene products of RVFV were expressed in baculovirus vectors. Partial sequence data for the L RNA of RVFV were obtained.

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DIAGNOSIS AND PREVENTION OF INFECTION BY PHLEBOTOMUS FEVER GROUP VIRUSES

ANNUAL/FINAL REPORT

DAVID H.L. BISHOP

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TABLE OF CONTENTS

I. SUMMARY

The objectives of the contract were to develop new diagnostic procedures and new vaccine strategies pertinent to selected Phlebotomus fever (PHL) group viruses, viruses that constitute the *Phlebovirus* genus of the negative-sense RNA virus family Bunyaviridae [Bishop, 1990]. This genus of viruses includes members that are known human pathogens and that are of consequence to military and civilian personnel in particular regions of the world [Bishop & Shope, 1979].

The development of new diagnostic tools for the specific identification of PHL group viruses was investigated using eukaryotic expression vectors and cDNA clones of particular phlebovirus genes. Two expression systems were studied, vaccinia and the insect-specific baculovirus expression vectors. It proved difficult to express the complete M RNA gene products of Punta Toro (PT) phlebovirus using either vector. Recombinant vaccinia viruses were made that expressed part of the PT M RNA, producing the PT glycoproteins G1 and G2 [Matsuoka *et al.*, 1988, 1989]. The expressed products were characterized. The expression of PT S RNA gene products [Overton *et al.*, 1987] using *Autographa californica* nuclear polyhedrosis virus [AcNPV] baculovirus vectors in insect cells was achieved. Expression of Hantaan N protein using similar strategies was undertaken in conjunction with USAMRIID staff and the product tested as a potential diagnostic reagent (Schmaljohn *et al.*, 1988).

The objective of developing new vaccine strategies for PHL group viruses involved the characterization of an attenuated, candidate vaccine derivative of Rift valley fever [RVF] phlebovirus [the M12 mutant of RVFV isolate ZH548]. This candidate vaccine was previously developed by staff of the Principal Investigator working at USAMRIID [Caplen *et al.*, 1984]. The complete sequence of the viral M RNA of the M12 RVFV derivative was obtained [Takehara *et al.*, 1989]. The M RNA of the RVFV M12 derivative was expressed in baculovirus vectors and the products characterized [Takehara *et al*, 1990]. The sequence of the S RNA of the M12 derivative of RVFV (Giorgi *et al.*, 1991) has also been obtained and compared with new data for Toscana (TOS) phlebovirus and previously published data of PT S RNA (Ihara *et al.*, 1984), Sicilian sandfly fever virus S RNA (SFS, Marriott *et al.*, 1990) and that of Uukuniemi virus (UUK, Simons *et al.*, 1990). The homologies of these sequences have identified conserved and variable regions of the genes and their gene products. The S gene products of RVFV have been expressed in baculovirus vectors. Partial sequence data for the L RNA of the

M12 mutant of RVFV have been obtained and a novel procedure developed to identify recombinant virus genotypes by hybridization techniques.

II. REPORT

A. Introduction

Our analyses have been directed towards developing new strategies for the development of phlebovirus vaccines and diagnostic reagents. Initial studies, supported by prior U.S. Army Medical Research and Development contracts, were aimed at characterising the genome and structural components of phleboviruses. With the demonstration of a tripartite RNA genome for phleboviruses, genetic analyses were then undertaken to delineate the coding strategies of the viral RNA species and to determine if recombinant viruses could be obtained and used for vaccine purposes. By analyses of intertypic reassortant PT viruses we initially showed that the viral $7x10^5$ dalton small [S] RNA species codes for the viral $26.9x10^3$ dalton nucleocapsid [N] protein. The results of cloning and sequencing the S RNA of PT phlebovirus [Ihara *et al.*, 1984] confirmed these data and showed that an open reading frame in the *viral-complementary* S RNA sequence coded for N [S mRNA]. A second open reading frame in the *viral-sense* strand, coding for a $29.1x10^3$ dalton non-structural protein, NS_S [Ihara *et al.*, 1984], was also identified. The two subgenomic PT S induced mRNA species [one viral-complementary, the other viral-sense] were characterized with regard to their 3' and 5' end sequences and rates of synthesis in infected cells [Ihara *et al.*, 1985a; Emery & Bishop, 1987]. These results have been confirmed by others working on SFS (Marriott *et al.*, 1990) and UUK viruses (Simons *et al.*, 1990) as well as studies supported by this contract (see below) on the S RNA of RVFV and TOS viruses (Giorgi *et al.*,1991).

The results of cloning and sequencing the $2x10^6$ dalton middle [M] size RNA of PT virus [Ihara *et al.*, 1985b] demonstrated that the PT M RNA codes for the $50\text{-}70x10^3$ dalton viral glycoproteins, G1 and G2. A $30x10^3$ dalton non-structural protein, NS_M, that constitutes the amino terminal end of the PT glycoprotein precursor was also identified. The data showed that the order of the PT M gene products was NS_M-G1-G2. The PT M viral-complementary mRNA species were characterized [Ihara *et al.*, 1985a]. The results obtained for the PT M RNA correlated with the data reported by Collett and associates [1985] for RVFV ZH501 M RNA and its gene products [RVFV M gene order: NS_M-G2-G1]. Results obtained by support from this contract (see below) described the M RNA sequence of the M12 derivative of ZH548 (Takehara *et al.*, 1989). Staff at USAMRIID

obtained the data for the parent ZH548 virus (Takehara *et al.*, 1989). Apart from identifying a sequencing (misreading) mistake in the RVFV ZH501 data, the results of the comparisons documented a number of nucleotide and amino acid differences between ZH501 and ZH548 (parent) and ZH548 parent and the M12 derivative.

It can be concluded that the phlebovirus $3x10^6$ dalton large [L] RNA species codes for the $200x10^3$ dalton large protein [a putative transcriptase component] found in viruses. Partial sequence data on the RVFV L RNA indicate that this assumption is correct.

The genetic studies, including interference assays, reported from the work conducted in the previous contract, indicated that although intertypic reassortant viruses could be obtained, heterotypic virus interactions were not demonstrable (i.e., heterotypic phleboviruses did not interfere with each other and did not reassort their genomes in dual virus infections). Although not all phleboviruses were tested for genetic interactions, the results did not hold out much hope for this approach for vaccine development. Consequently, alternative approaches have been investigated involving the development of candidate, including subunit, phlebovirus vaccines (see below). In addition, research was directed towards the development of new and specific diagnostic reagents for phleboviruses of military importance and means to identify virus recombinants using specific nucleic acid probes.

B. Results.

Expression of the PT N and NS proteins using baculovirus expression vectors

An essentially complete DNA copy of the ambisense S RNA species of PT phlebovirus [Ihara *et al*, 1984a] has been inserted in either orientation into *Autographa californica* nuclear polyhedrosis baculovirus (AcNPV) in lieu of the 5' coding region of the AcNPV polyhedrin gene [Overton *et al.*, 1987]. The recombinant viruses were generated using what proved to be proficient, but non-optimal expression vectors [e.g., pAcRP6, see below]. The two types of recombinant viruses were used to infect *Spodoptera frugiperda* cells and the expressed PT viral proteins characterised (Overton *et al.*, 1987). Recombinant AcNPV having the S DNA in one orientation expressed PT virus N protein in amounts estimated to represent some 50% of the infected cell extracts, whereas recombinants with the PT S DNA in the other orientation expressed the PT virus NS_S protein in significantly lower quantities. Antisera that were monospecfic

with respect to each of the two PT proteins virus were raised in mice using the corresponding *S. frugiperda* infected cell extracts and were employed to identify N and NS_S proteins in PT virus-infected Vero cells. These studies demonstrated a low amount of PT NS_S protein in virions and virion-derived nucleocapsids (Overton *et al.*, 1987).

The results reported previously by this laboratory have been extended by insertion of each individual gene in a more efficient expression vector developed in our laboratory [pAcYM1, Matsuura *et al.*, 1987]. The level of expression of the PT N protein was similar to that obtained with the pAcRP6 vector, however the level of expression of the NS_S protein was considerably enhanced, approaching that of the N protein.

Expression of PT virus glycoproteins from partial cDNA clones inserted into a vaccinia vector

Partial cDNA clones of the PT M RNA were inserted into the genome of vaccinia virus so that the PT gene products were under the control of an early vaccinia promoter. The PT sequences included in the constructs represented the entire G1 and G2 coding sequences preceeded by some 24-37 upstream amino acids [depending on the constructions]. Recombinant vaccinia viruses were prepared using vaccinia plasmid pSC11 and transfection of CV-1 cells previously infected with strain IHD-J of vaccinia virus. Recombinant viruses were selected by the appropriate procedures. In CV-1 cells the recombinants expressed glycosylated derivatives of both PT G1 and G2 proteins. In Hela T4 cells the glycoproteins were identified in the Golgi apparatus [Matsuoka *et al.*, 1988, 1989].

The sequence of the M RNA of the M12 candidate vaccine of RVFV

Analyses of cDNA clones of the M RNA of the ZH501 strain of RVFV have been reported by Collett and associates (1985). In order to characterize the candidate RVFV vaccine that was produced from the related ZH548 RVFV [Caplen *et al.*, 1984], we have sequenced the M RNA of the M12 derivative. The sequence was obtained by analyses of λ cDNA clones (Takehara *et al.*, 1989). Based on these cDNA sequence analyses the RVFV M12 M RNA was deduced to be 3885 nucleotides long (mol. wt. 1.38×10^6, base composition: 27.3% A, 27.2% U, 25.4% G, 20.1% C) with 3'- and 5'-terminal sequences that are complementary for

some 9 residues. The viral RNA codes in its viral-complementary sequence for a single long gene product [the viral glycoprotein precursor] that is comprised of 1197 amino acids (130,000 daltons). The gene product is abundant in cysteine residues but has few potential asparagine-linked glycosylation sites. It is initiated by a methionine codon at nucleotide residues 21-23. These data are similar to those reported for RVFV ZH501 M RNA. However, unlike the ZH501 sequence, there is a second AUG codon [residues 9-11] in the viral-complementary sequence *upstream* of the M12 M gene product. It is followed by valine and histidine codons then a translation terminator [TAA, residues 18-20]. Even discounting this region the 5' non-coding region of the RVFV M viral-complementary RNA is short (20 nucleotides); the 3'-noncoding sequence is much longer (271 nucleotides). No other large open reading frame has been identified in either the viral, or viral-complementary RVFV M RNA sequences (Takehara *et al.*, 1989).

Limited amino-terminal sequence analyses of the ZH501 RVFV glycoproteins indicated the gene order and potential cleavage sites in the glycoprotein precursor. The data suggested the existence of a $23x10^3$ dalton polypeptide (designated NS_M) in the glycoprotein precursor that precedes the RVFV G2 and G1 proteins (i.e., gene product order: NS_M-G2-G1). Examination of the sequence of the M12 gene product indicates that the ZH548 and ZH501 sequences are closely related and that the amino terminal sequences of the viral glycoproteins are conserved between them. As shown for PT and the RVFV ZH501 glycoproteins there are multiple hydrophobic sequences in the M12 M gene product, including a 22-28 amino acid carboxy-proximal, hydrophobic region (G1). This hydrophobic sequence is followed by a 11-amino acid-terminal sequence that contains 3 charged (basic) amino acids. The size and constitution of the carboxy-terminal region is consistent with a transmembranal and anchor function for this glycoprotein in the viral envelope. Other regions of the glycoprotein precursor contain sequences of amino acids with a predominantly hydrophobic character. Their functions are unknown.

Comparison has been made between the reported sequence of ZH501 M RNA and its gene product and those of the M12 mutant of ZH548 and the parent ZH548 virus. The number of nucleotide and amino acid differences have been quantified (Takehara *et al.*, 1989).

Expression of the M RNA of the M12 candidate vaccine of RVFV using baculovirus vectors

A cDNA corresponding to the complete coding region of the M RNA of the M12 mutant of RVFV ZH548 was inserted into the baculovirus transfer vector pAcYM1 (Takehara, *et al.*, 1990). By comparison with the parent RVFV, the M RNA of the M12 mutant has a new small open reading frame (ORF1) upstream of the one that initiates the precursor of the viral glycoproteins (ORF2, gene order: NS_M-G2-G1). A derivative of the M12 cDNA was prepared from which most of the upstream sequences (including a polyT track and ORF1) were removed. Other cDNA constructs were made from this derivative, constructs in which most of the G1 sequences were also removed, or most of the NS_M coding sequences, or all of the NS_M and most of G2 coding sequences. Each RVFV M cDNA construct was inserted into a pAcYM1 transfer vector and recombinant baculoviruses were produced (RVM1-5). The derived viruses were employed to study the expression and properties of the RVFV glycoproteins in *S. frugiperda* insect cells. For each recombinant virus evidence was obtained which indicated that the RVFV glycoproteins were produced and processed in the insect cells (Takehara, *et al.*, 1990). Although four of the recombinants gave low expression levels of the RVFV glycoproteins, for the vector that made only the G1 product, the expression level was significantly higher. Immunofluorescence analyses established that the RVFV glycoproteins were present both at intracellular locations and on the surface of the recombinant baculovirus infected insect cells.

Comparison of RVFV S and TOS S cDNA sequences with those of PT, SFS and UUK viruses

The sequences and coding strategies of the S RNAs of TOS and the M12 derivative of RVFV ZH548 have been determined from cDNA clones and compared to the previously published sequences of PT, SFS and UUK viruses (Giorgi *et al.*, 1991). All five viruses exhibit an ambisense coding strategy for their S RNA species, i.e., the NS_S protein is encoded in the 5' half of the viral RNA, the N protein is encoded in the sequence complementary to the 3' half. The terminal nucleotides at the ends of the S RNAs of the five viruses are comparable through 13-14 residues. The 3' and 5' ends of these S RNAs have inverted complementary compositions. Three phleboviruses (TOS, SFS, RVFV) exhibit comparable G-rich, centrally located intergenic sequences, albeit of different lengths. These sequences have a number of similar motifs at, or immediately following the end of the coding regions, motifs that may be involved in their S mRNA transcription

termination processes. The other two viruses (UUK, PT) have AT-rich intergenic sequences that have the potential to form secondary structure. They lack the G-rich sequences or particular sequence motifs recognised in the other three virus RNAs. The deduced sizes of the TOS and RVFV N proteins are 27,704 and 27,430 kDa (respectively). Their NS_S proteins are 36,677 and 29,903 kDa (respectively). When aligned, the deduced sequences of the N proteins of the five viruses exhibit homologies ranging from 54% to 30%. The order of homology to RVFV N protein is PT>TOS>SFS>UUK; to TOS N protein it is PT≅RVF>SFS>UUK. The sequences of the NS_S proteins are less similar, with values ranging from 30% to <17%. The order of homology to RVFV NS_S is SFS>PT>TOS>UUK. Due to these more distant relationships, the homologies to TOS NS_S protein are less clear.

The individual N and NS_S genes of RVFV S RNA have been inserted into baculovirus expression vectors and the proteins expressed to high levels.

Preliminary sequence analyses of the RVFV L RNA

Approximately 2kb of sequence of the L RNA of the M12 derivative of ZH548 have been obtained from analyses of λ cDNA clones. The data indicate the existence of a single long open reading frame in the viral-complementary RNA.

Development of specific genetic probes to differentiate closely related viruses

Viruses that are very closely related to each other (at the genetic and gene product level), even though they may differ in phenotype (virulence, vector preferences, etc.) can prove difficult to distinguish. A chimaeric genetic probe has been developed and tested to distinguish the S RNAs of two closely related bunyaviruses, snowshoe hare (SSH) and La Crosse (LAC) viruses. The technique is applicable to other SSH and LAC RNA species and other closely related viruses (including phleboviruses such as RVFV).

Out of a total of 982-984 nucleotides for the S RNA species of prototype SSH and LAC viruses, there are 114 nucleotide differences. Of these, 6 are additions or deletions, 108 are nucleotide substitutions (Akashi & Bishop, 1983). Because of this similarity it is not possible to distinguish the S RNAs of parent or progeny viruses by conventional hybridization procedures using cloned DNA representing

the entire genome. A similar problem pertains for the SSH and LAC M and L RNA species.

It has been observed that the sequence differences between SSH and LAC S RNA species are not randomly dispersed in the viral genome (Akashi & Bishop, 1983). Two regions of the S sequences of these viruses exhibit more variation than elsewhere. In view of this observation and since the individual divergence at either site was not extensive, chimaeric oligonucleotide sequences representing these regions were synthesized chemically. The oligonucleotides were cloned into the plasmid pSPT18. The LAC oligonucleotide probe was synthesized so that it consisted of an in-tandem sequence representing LAC viral-complementary S residues 56-83,784-815. The corresponding SSH probe was represented by SSH viral-complementary residues 55-80,781-814, also in tandem. The oligonucleotides were backcopied and blunt-end cloned into a plasmid (Nolan *et al.*, 1989).

Viral-complementary RNA probes were synthesized from the cloned DNAs in the presence of [α-32P]UTP (800 Ci/mmol) using T7 RNA polymerase after linearization of plasmid DNA with *Hind*III. Alternatively DNA samples were cleaved with *Eco*RI and transcribed with SP6 polymerase. The RNA products, representing the viral-complementary RNA sequences (to hybridize to viral RNA) were purified by phenol extraction and gel filtration prior to use in Northern analyses. Cytoplasmic RNA preparations of SSH, LAC and the six possible SSH-LAC reassortant viruses were prepared, resolved by electrophoresis in 1% agarose gels containing 10 mM methylmercury, transferred to Hybond-N membranes, fixed to the membrane and probed with radioactive RNA transcripts. The results of the analyses (Nolan *et al.*, 1989) unambiguously demonstrated that in all cases the SSH S RNA could be distinguished from that of LAC virus using the oligonucleotide probes but not when full-length cDNA probes were used.

C. Summary

The sequences of the S and M RNA species of the M12 derivative of RVFV ZH548 have been obtained and compared to those of related phleboviruses to identify regions of conservation and divergence. The genes encoded by each of the RNA species have been inserted into baculovirus expression vectors and the properties of the expressed products have been investigated. The glycoproteins are processed and transported to the infected cell surface. Some sequence information

for the L RNA of RVFV ZH548 M12 has been obtained. A novel procedure to use chimaeric probes to distinguish closely related viruses has been developed.

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